STRINGENT FACTOR BINDS TO *ESCHERICHIA COLI* RIBOSOMES ONLY IN THE PRESENCE OF PROTEIN L10

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1. Introduction

The stringent factor isolated from *E. coli* has been characterized in terms of the details of the reaction it catalyzes, as well as defining the components necessary for the reaction to proceed [1–3]. Recently, it was shown that the stringent factor remains bound to the 70 S ribosomes during the synthesis of guanosine-5′-diphosphate-3′-diphosphate (ppGpp) and guanosine-5′-triphosphate-3′-diphosphate (pppGpp), while the uncharged tRNA actually cycles on and off the ribosomes with each ATP hydrolysed [4]. The study we report here was designed to identify specific proteins involved in the stringent factor binding site on the ribosome. It was shown earlier that the presence of ribosomal proteins L7 and L12 on the 50 S subunit is unnecessary for both stringent factor activity and binding to ribosomes [3]. Here, we investigated a series of ribosome core particles deficient in other defined proteins and groups of proteins. We demonstrated which protein must be removed in order to eliminate the stringent factor activity and binding and whose re-addition restored the activity.

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2. Materials and methods

2.1. *Materials*

The stringent factor was isolated from *E. coli* strain CGSC 2834/a and purified by hydroxyapatite and DEAE-cellulose chromatography, and glycerol gradient centrifugation [3]. 70 S ribosomes, 50 S and 30 S ribosomal subunits were isolated from *E. coli* as previously described [5]. The 50 S core particles and split proteins were prepared as described by Hamel et al. [6]. The P_0 core particles lack ribosomal proteins L7 and L12; the P_{37} core particles lack proteins L7, L10, and L12; P_{0-37} core particles lack proteins L7, L10, L11 and L12; and the P_{0-37} split proteins are predominantly L10 and L11 as described earlier [7] and confirmed for the preparations used here. Poly(U) and $tRNA^{Phe}_{yeast}$ were purchased from Boehringer/Mannheim; [α-^{32}P]GTP (sp. act. 20 mCi/mol) and [^{32}P]NaH_2PO_4 (sp. act. 2 Ci/mmol) were from New England Nuclear Corp., Boston. Polyethylene-imino cellulose thinlayer plates (Cel 300 PEI) were purchased from Macherey-Nagel Co., Düren, Germany; X-ray film CR/R-54) from Kodak, Rochester. Sodium [^{3}H]borohydride (sp. act. 6–40 Ci/mmol) was obtained from Amersham, Braunschweig, Germany. Benzoylated DEAE-cellulose was purchased from Schwarz Bioresearch. A_{260} units were calculated by measuring absorbance at 260 nm with

a 1 cm cuvette against 10 mM Tris–HCl (pH 7.4): 1 A_{260} unit of 70 S ribosomes = 25 pmol, 1 A_{260} unit of 50 S subunits = 39 pmol, 1 A_{260} unit of 30 S subunits = 67 pmol, 1 A_{260} unit of tRNA = 1.5 nmol.

2.2. *Preparation of [^{3}H]stringent factor and [^{32}P]tRNA*

[^{3}H]stringent factor (0.5–5.0 Ci/mol) was prepared by the reductive methylation method [8], using formaldehyde and [^{3}H]borohydride [2]. [^{32}P]tRNA was prepared by growing yeast cells (*Saccharomyces fragilis*) with 5–10 mCi of [^{32}P]NaH_2PO_4 [9]. [^{32}P]tRNA (5 Ci/mol) was isolated and purified by benzolylated DEAE-cellulose column chromatography [10].

2.3. *Reconstitution of core particles*

Ribosomal core particles were reconstituted with split protein fractions by simply incubating the two components together for 5 min at 0°C [7]. The molar ratio of split proteins to core particles was approximately 2:1. The particles were then isolated from the reconstitution mixture by centrifugation in an SW 56 rotor (18 h; 55 000 rpm; 4°C) through 7% sucrose made up in 10 mM Tris–HCl (pH 7.4), 10 mM $MgCl_2$, 50 mM NH_4Cl and 2 mM dithiothreitol. The pelleted particles were then resuspended in this buffer and stored in liquid nitrogen until used.

2.4. *Assay for synthesis of guanosine polyphosphates*

The 50 μl reaction mixtures contained (unless otherwise indicated) 8.25 pmol of 50 S subunits, 9 pmol of 30 S subunits, 1.0 μg $tRNA^{Phe}_{yeast}$, 2.5 μg poly(U), 4 mM ATP, 20 mM Tris–HCl (pH 7.8), 20 mM Mg-acetate, 2 mM dithiothreitol, 40 mM NH_4Cl, 0.4–0.5 μg of purified stringent factor and 0.4 mM [α-^{32}P]GTP, which was added last to start the reactions. The reaction mixtures were incubated 1 h at 37°C; incubation was stopped by the addition of 1 μl of 88% formic acid. A precipitate was removed by centrifugation and aliquots were applied to PEI thin-layer plates. The plates were developed with phosphate buffer and exposed to X-ray film [11]. Radioactive spots were scanned in a thin-layer scanner (BF-Vertriebs GmbH, Karlsruhe, Germany) to determine the percent of GTP converted into pppGpp and ppGpp.

3. Results and discussion

Ribosomal 50 S subunits were depleted of specific groups of proteins by combinations of ethanol-high salt and temperature treatments. The resultant core particles were then tested for their activity in (p)ppGpp synthesis, in the presence of 30 S subunits and stringent factor. In the experiment shown in fig.1 core particles lacking proteins L7 and L12 only (P_0 cores) retain 50–80% of the activity of intact 50 S subunits over the concentrations examined. If in addition to proteins L7 and L12 we remove proteins L10 and L11 (P_{0-37} cores) or just protein L10 (P_{37} cores) the stringent factor activity is completely lost. Figure 1 also illustrates the relationship between the amount of these various core particles and the activity remaining in the standard 1 h assay for the conversion of GTP to (p)ppGpp. Increasing the molar amount of inactive core particles by a factor of 5 (over the amount of 50 S subunits necessary to reach a plateau

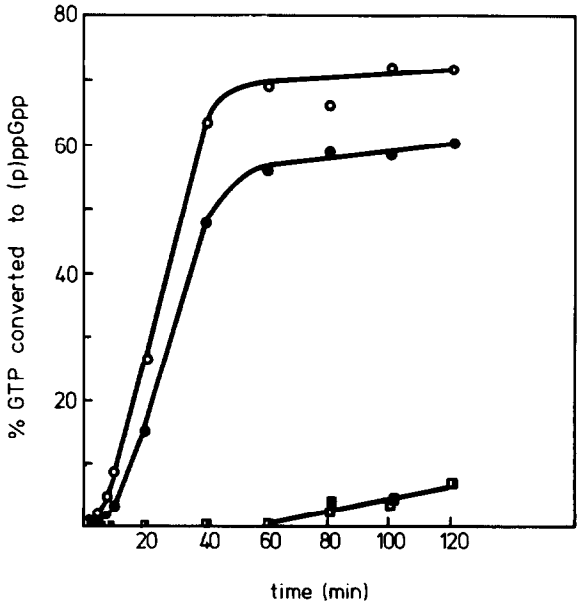

Fig.1. Synthesis of (p)ppGpp with 50 S subunits and core particles. Various amounts of 50 S subunits or core particles were mixed with 10 pmol of 30 S subunits, incubated 1 h in the reaction mixture as described in Materials and methods, and the amount of GTP converted to pppGpp and ppGpp determined. (○–○), 50 S subunits; (●–●), P_0 core particles; (■–■), P_{37} core particles; (□–□) P_{0-37} core particles.

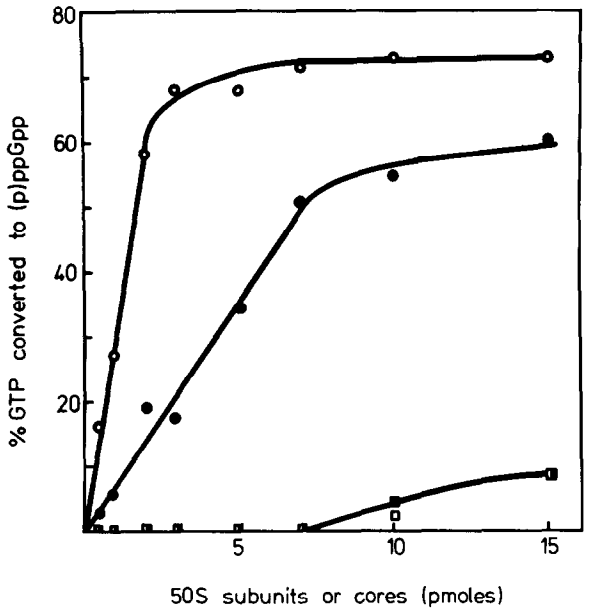

Fig.2. Kinetics of (p)ppGpp synthesis with ribosomes depleted of various 50 S subunit proteins. 10 pmol of 50 S subunits or indicated core particles were mixed with 10 pmol of 30 S subunits and incubated as described in Materials and methods. Incubation was started with the addition of [α-^{32}P] GTP. At the times indicated 5 μl aliquots were withdrawn and 1 μl of 8.8% formic acid was added to them to stop the reaction. The percentage of GTP converted to pppGpp and ppGpp was determined as described in Materials and methods. (○–○), 50 S subunits; (●–●), P_0 core particles; (■–■), P_{37} core particles; (□–□), P_{0-37} core particles.

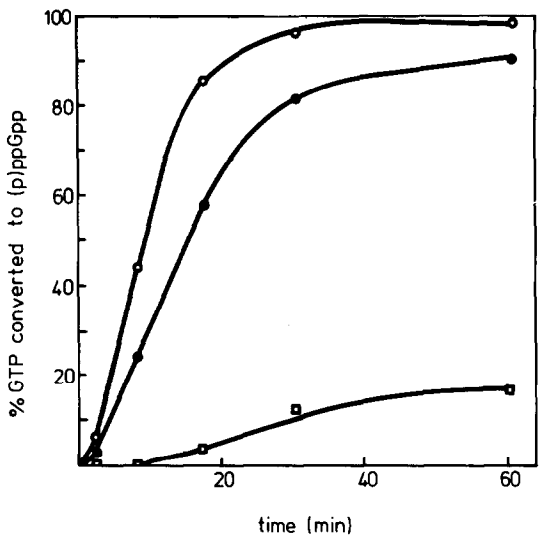

Fig.3. Kinetics of (p)ppGpp synthesis with reconstituted 50 S ribosomal subunits. 10 pmol of 50 S subunits or indicated core particles and split proteins were mixed with 10 pmol of 30 S subunits, incubated and assayed for conversion of GTP to (p)ppGpp in the 50 μl reaction mixture described in Materials and methods. Other conditions were as described in the legend to fig.2. (○–○), 50 S subunits; (□–□), P_{37} core particles; (●–●), P_{37} core particles plus P_{0-37} split proteins.

in the activity) does not yield any significant increase in their activity.

If we use an amount of 50 S (or core particles) sufficient to reach a plateau in the activity during the standard 1 h reaction conditions, we can then examine the kinetics of GTP conversion mediated by the stringent factor. These results are shown in fig.2. The reaction is essentially complete after 1 h. Furthermore, the P_0 cores here have activity in this assay approaching that of the intact 50 S subunits, as had been described earlier [3]. The removal of protein L10 (or L10 and L11), however, leaves very little stringent factor activity over the time period studied.

The obvious control to the experiments described above of removing just enough proteins from the ribosome to cause a loss of activity, is to add back just sufficient proteins to restore activity to the core particles. The results of such an experiment are shown in fig.3. Although the P_{37} core particles (in presence of 30 S subunits) have very little activity, supplementing them with protein L10 (P_{0-37} split proteins: L10 and L11 [5]) restores their activity essentially to the level of intact 50 S subunits. (The activities are a bit higher here overall than in the experiments shown in figs.1 and 2, as fresh particles were used which had not been frozen for any length of time, and hence had not lost activity.) Thus, the inactivity of the P_{37} and P_{0-37} cores is not due to irreversible denaturation, but due to the complete absence of a particular protein. These experiments show the necessity of protein L10 for ribosomes to carry out the stringent factor reactions. The experiment described in table 1 extends these results to show the necessity of protein L10 for actual binding of the stringent factor to ribosomal core particles. The P_{37} cores, depleted of proteins L7, L10 and L12, bind very little [^{3}H] stringent factor and hence, a low level of uncharged tRNA, whereas the addition of protein L10 (via P_{0-37} split proteins, L10 and L11) to these particles

Table 1
Binding of [^{3}H]stringent factor and [^{32}P]tRNA to reconstituted 50 S ribosomal subunits

Ribosomal subunits	[^{3}H]Stringent factor (pmol) Bound per pmol ribosomes	[^{32}P]tRNA (pmol)	GTP to (p)ppGpp (%)
50 S + 30 S	0.17	0.24	83.1
P_{37} core + 30 S	0.03	0.12	8.1
P_{37} core + P_{0-37} split + 30 S	0.14	0.22	64.5

Reaction mixtures (final vol. 250 μl) which contained 300 pmol 50 S subunits or equivalent particles, 300 pmol 30 S subunits, 200 μg poly(U), 40 mM Tris–HCl (pH 7.8), 10 mM Mg-acetate, 4 mM dithiothreitol, were incubated 10 min at 30°C, then cooled to 4°C. 400 pmol each of [^{3}H]stringent factor and [^{32}P]tRNA were added (final vol, 500 μl). Final buffer (A) conditions: 20 mM Mg-acetate, 2 mM dithiothreitol. The mixtures were incubated 30 min at 4°C, then centrifuged through 1.6 ml of 5% sucrose in buffer (A) for 3.5 h at 160 000 × *g* in a Beckman Ti40 rotor. The ribosomal pellets were rinsed with 2 ml of buffer (A), then resuspended in 50 μl of buffer (A). The solution was measured at 260 and 280 nm, the yield was 125 pmol assuming that the solution contained mostly 70 S ribosomes. The complexed ribosomes were analyzed for ^{3}H and ^{32}P radioactivity, and for (p)ppGpp synthesizing activity (see Materials and methods).

increases their ability to bind stringent factor to essentially the level of intact 50 S subunits. There is also a concomitant increase in the ability of the particles to bind uncharged tRNA. The residual binding of tRNA by P_{37} cores (table 1, line 2) may reflect binding to a site non-identical to the A site.

The fact that this is specific binding is shown by the ability of the bound stringent factor to stimulate the conversion of GTP to (p)ppGpp. Thus, although protein L10 may or may not be the exact binding site for the stringent factor, its presence on ribosomal core particles is mandatory for such particles to bind the stringent factor, and subsequently to carry out the stringent factor mediated conversion of GTP to (p)ppGpp.

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